PATENT

Murine Calicivirus

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims benefit of priority to Provisional U.S. Patent Application Serial Number 60/440,016, filed January 14, 2003, which application is hereby incorporated herein by reference in its entirety.

REFERENCE TO GOVERNMENT GRANT

This invention was made with government support under Grant No. RO1 AI49286. The United States government may have certain rights in the invention.

BACKGROUND

The *Caliciviridae* are a family of positive-sense, single-stranded RNA viruses with a 7-8kb genome that are divided into 4 distinct genera and further subdivided into genogroups. The genera Norwalk-like viruses, together with the closely related Sapporo-like viruses, recently renamed *Noroviruses* and *Sapoviruses* (Mayo, M.A., *Arch. Virol.* 147:1655-1656, 2002), make up human caliciviruses (Kapikian, A.Z. et al., *J. Virol.* 10:1075-1081, 1972; Jiang, X. et al.,*Science* 250:1580-1583, 1990; Jiang, X. et al., *Virol.* 195:51-61, 1993; Hardy, M.E. et al., *Virus Genes* 12:287-290, 1996). *Noroviruses* are responsible for more than 90% of all cases of non-bacterial

epidemic gastroenteritis (Kapikian et al., 1972; Kapikian, A.Z. et al., Chapter 25 in *Fields Virology*, Fields, B.N. et al., Eds., 1996; Pang, X.L. et al., *Pediatr. Infect. Dis. J.* 18:420-426, 1999; Pang, X.L. et al., *J. Infect. Dis.* 181(Supp. 2):S288-S294, 2000; Fankhauser, R.L. et al., *J. Infect. Dis.* 178:1571-1578, 1998; Glass, R.I. et al., *J. Infect. Dis.* 181(Supp. 2):S254-S261, 2000; Hedlund, K.O. et al., *J. Infect. Dis.* 181(Supp. 2):S275-S280, 2000; Koopmans, M. et al., *J. Infect. Dis.* 181(Supp. 2):S262-S269, 2000; Inouye, S. et al., *J. Infect. Dis.* 181(Supp. 2):S270-S274, 2000). There are no current therapeutic drugs or vaccines for these important human pathogens. *Sapoviruses* are typically associated with sporadic cases of pediatric gastroenteritis (Pang et al., 1999; Pang et al., 2000). Two other calicivirus genera, *Vesiviruses* and *Lagoviruses*, contain animal viruses exclusively. Calicivirus genomes typically contain a large 5' open reading frame (ORF1) encoding a nonstructural polyprotein, followed by ORF2 encoding a single capsid protein. ORF2 is either in frame with ORF1 or present as an independent ORF. While the 5' end of ORF1 shows extensive sequence diversity, the remainder of ORF1 contains motifs arranged in a specific order conserved between caliciviruses and picornaviruses. ORF3, encoding a basic protein, is present at the 3' end of the genome preceding a poly-A tract (Clarke, I.N. et al., *J. Infect. Dis.* 181(Supp. 2):S309-S316, 2000).

DESCRIPTION OF THE FIGURES

Figure 1: Passage of a new pathogen by intracranial inoculation in RAG/STAT-/- and IFNαβγR-/- mice.

The unknown pathogen was passaged into RAG/STAT-/- and IFNαβγR-/- mice and caused lethal disease within 30 days of inoculation (A), characterized histologically by meningitis (C), vasculitis of the cerebral vessel (D), and encephalitis (E) compared to mock-infected brain (B). (B, C) RAG/STAT-/- mice; (D, E) IFNαβγR-/- mice. Brain homogenate from an infected RAG/STAT-/- mouse was passed into 129 wild-type mice (A) and sera of these mice harvested 35 days later tested negative for mycoplasma, Sendai virus, reovirus type 3, Theiler's mouse encephalomyelitis virus (GDVII strain), lymphocytic choriomeningitis virus, pneumonia virus of mice, minute virus of mice, mouse hepatitis virus, ectromelia virus, epizootic diarrhea of infant mice, mouse cytomegalovirus, polyoma virus, K virus, orphan parvovirus, and mouse adenovirus.

Figure 2: Sequencing and phylogenetic analysis of the MNV-1 genome.

A) Double-stranded cDNA (dsDNA) from the brain of an infected IFNαβγR-/- mouse at passage 2 (Fig. 1) was prepared, digested with restriction enzymes, and ligated to adaptors containing PCR primer sequences to generate "tester" nucleic acids. dsDNA lacking linkers was prepared concurrently from a control brain to generate "driver" nucleic acids. Serial rounds of subtractive hybridization of tester in the presence of excess driver followed by PCR amplification of tester-specific sequences were performed to generate difference products (DP) one through four (DP1-DP4). DP3 and DP4 were cloned into pGEMT (Promega, Madison, WI), sequenced, analyzed using BLAST, and clones (1-8, Fig. 2A) homologous, but not identical, to calicivirus sequences were identified that spanned the Norwalk virus genome. Sequences within RDA clones (indicated by asterisks) were used to clone and sequence five fragments (a', b', c', d', e', Fig. 2A) of the MNV-1 genome after PCR or 5' and 3' RACE (Marathon cDNA amplification kit, Clontech, Palo Alto, CA). The 5' end of the genome was difficult to clone and consequently the first 15 nucleotides are based on a single sequence, while the remaining sequence has at least a 10-fold redundancy. This may explain why there is no start codon close to the 5' end as is expected based on comparison with other *Noroviruses*. B) Schematic of the final 7726 bp MNV-1 genome sequence with predicted open reading frames (ORFs). The locations of amino acid motifs in ORF1 are indicated: 2C helicase: GXXGXGKT (SEQ ID NO: 50); 3C protease: GDCG (SEQ ID NO: 51); 3D polymerase: KDEL (SEQ ID NO: 52), GLPS (SEQ ID NO: 53), YGDD (SEQ ID NO: 54). The putative S and P domains of the ORF2 encoded capsid protein were identified based on sequence alignments with Norwalk virus. AAA: 3' poly-A tail. C) Alignment of the complete MNV-1 genome with complete genomes of representative members of the four *Caliciviridae* genera and members of the most closely related virus family, the *Picornaviridae*. Specific members were chosen based on the 2000 taxonomy study by Green *et al.* (J Infect Dis '00 v. 81 p. S322). D) Alignment of the capsid protein sequence of MNV-1, done as in C. Note that the alignments in C and D were confirmed using other algorithms (data not presented).

Figure 3: Sequence variability of MNV-1.

A) All variable nucleotides within ORF1 and ORF2, based on sequence analysis of multiple clones of the entire MNV-1 genome, are depicted. These nucleotides had 20% or less variability between clones. B) Sequences of individual clones spanning nucleotides 1767 to 1893 (solid box on ORF1 in panel A), with variable positions highlighted with arrowheads SEQ ID Nos:21-48).

The consensus sequence of MNV-1 is shown at the bottom (bold type) (SEQ ID NO:49), with variable nucleotides highlighted by arrowheads.

Figure 4: Purification and pathogenicity of MNV-1.

MNV-1 was purified from an infected IFNαβγR-/- mouse brain homogenate by CsCl density gradient centrifugation. As a control, mock-infected mouse brain homogenates were processed similarly. (A) Determination of the average buoyant density of genome-containing MNV-1 particles. Dialyzed gradient fractions were analyzed by MNV-1 specific RT-PCR (Titanium one-step RT-PCR kit, Clontech, Palo Alto, CA) and products were separated on a 1% agarose gel. Primers were chosen in ORF1 to yield an expected product of 184 bp (indicated by the asterisk). (B) MNV-1 virions visualized by EM. Samples were absorbed onto formvar/carbon-coated grids for 1 min. The grids were washed in dH_2O, stained with 2% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) for 1 min, and air dried prior to viewing on a JEOL 1200EX transmission electron microscope (JEOL USA, Peabody, MA). (C) Survival of RAG/STAT-/- mice infected i.c. with unpurified, or purified MNV-1, as well as gradient fractions from mock-infected brain. The P values for mock versus infected mice are indicated. Statistical analyses were performed using GraphPad Prism software.

Figure 5: IFNαβ or IFNγ receptors and STAT1 are required to protect from lethal MNV-1 challenge.

A MNV-1 stock was prepared as a brain homogenate from 17 IFNαβγR-/- mice inoculated i.c. three days previously with brain homogenate from a passage 2 (Fig. 1) mouse. Infected brains were homogenized in sterile PBS and filtered through a 0.2μm filter. Brains from five IFNαβγR-/- mice inoculated i.c. with uninfected brain tissue were used to generate a mock virus stock. Mice of various strains were inoculated with MNV-1 or mock-inoculated using 10 μl intracerebrally (ic), 25 μl intranasally (in), or 25 μl perorally (po). A number of mouse strains did not show increased mortality compared to wild-type 129 controls (A). The survival after inoculation with MNV-1 or mock virus is shown for IFNαβγR-/- mice (B), STAT1-/- mice (C), RAG/STAT-/- mice (D), and STAT1/PKR-/- mice (E). All p values for mock versus infected groups were ≤0.0001 except: IFNαβγR-/- i.n.: p=0.002; STAT1-/- i.n.: p= 0.097; and STAT1-/- p.o.: p= 0.034. Statistical analyses were performed with GraphPad Prism software.

Figure 6: Generation of MNV-1 virus-like particles.

A) Western blot analysis of cell lysates from High-Five cells infected with recombinant baculovirus expressing the MNV-1 capsid protein (see Example 9) or a control baculovirus expressing the LacZ cassette (negative control). Proteins were detected by ECL Plus after incubation with serum from a MNV-1 infected mouse followed by a HRP-labeled secondary antibody. The size of the molecular weight marker is indicated on the right. B)-D) Electron microscopy of negatively stained VLPs. Supernatants of High-Five cells infected with a control baculovirus expressing LacZ (B), recombinant baculovirus expressing the MNV-1 capsid protein (C), or VLPs purified from these supernatants (D) were stained with uranyl acetate and photographed at a magnification of 50,000X.

Figure 7: Reactivity of mouse serum against MNV-1 VLP supernatants or cell lysates by ELISA.

Supernatants of High-Five cells infected with recombinant baculovirus expressing the MNV-1 capsid protein or LacZ expressing control were coated on ELISA plates. A) Analysis of half-log serial dilutions of serum from MNV-1 infected mice or 129 wild type mice. B) Analysis of 1:10 dilution of several cages of STAT-/- mice. Each dot represents one mouse. Reactivity was assessed after incubation with a HRP-coupled secondary antibody and colorimetric detection at 405nm. Cages 1, 3, 4, 5 and 6 contained seronegative mice. Cages 2, 7, 8, and 9 contained seropositive mice.

Figure 8: Tissue MNV-1 RNA levels after infection via different routes.

Four IFNαβγR -/- mice were inoculated with MNV-1 i.c. (10μl), p.o. (25μl), or i.n. (25μl). Two mice were sacrificed at both 2 and 7 dpi and lung (Lu), intestine (Int), brain (Br) and feces were collected. RNA was extracted from each organ, and cDNA was synthesized and used (5ng) in triplicate real time PCR reactions. Primers specific to a 131 nucleotide region of ORF1 were used (sense=cagtgccagccctcttat (SEQ ID NO:19); antisense=gtcccttgatgaggagga (SEQ ID NO:20)). Signal was compared to a standard curve generated using a plasmid containing target sequences. Triplicate reactions were performed using GAPDH primers to verify equivalent amounts of starting template (not shown). The levels of virus RNA as $\log_{10}$ MNV-1 genome copies are shown (open bars = 2 dpi, solid bars = 7 dpi, *=undetectable levels).

Figure 9: Immunohistochemical staining of spleen secti ns from MNV-1 infected mouse. Formalin-fixed spleen sections from a STAT1-/- animal 3 days after p.o. inoculation with MNV-1 were stained with either immune polyclonal rabbit serum inoculated with bacterially expressed MNV-1 capsid protein (left panel), or with the preimmune serum from the same rabbit (right panel). Immunohistochemistry was performed with the PerkinElmer™ TSA™-Plus DNP (HRP) System, according to the supplied protocol. Primary antibodies were used at a 1:25 dilution. Positive cells are indicated by arrows.

Figure 10: Single copy sensitivity of MNV-1 cDNA detection by nested PCR assay. Nested PCR primers specific to a region of MNV-1 ORF2 were designed (outer-sense=gcgcagcgccaaaagccaat (SEQ ID NO:15); outer-antisense=gagtcctttggcatgctacccagg (SEQ ID NO:16); inner-sense=gccgccgggcaaattaacca (SEQ ID NO:17); and inner-antisense=ggcttaacccctaccttgccca (SEQ ID NO:18)). A) Multiple PCR reactions with either 1 or 10 copies of a plasmid containing the appropriate region of MNV-1 were performed. 3/4 and 4/4 reactions were positive for 1 and 10 copies, respectively. The expected size of the PCR product is 153bp. B) cDNA was generated from spleen tissue of 10 IFNαβγR-/- mice and 1 μg of each was used in nested PCR reactions (7/10 samples were positive). All water controls are negative.

DESCRIPTION

It has been discovered that mice doubly deficient in STAT1 and RAG2 (RAG/STAT) contained an infectious pathogen that caused severe encephalitis and could be serially passaged by intracerebral (i.c.) inoculation (Fig. 1). Lethal infection was associated with encephalitis, vasculitis of the cerebral vessels, meningitis, hepatitis, and pneumonia (Fig. 1 and data not shown). Disease was passed by filtered samples, suggesting the presence of a virus (Fig. 1A). Sera of 129 mice infected with the putative virus tested negative for an extensive panel of mouse pathogens (see legend of Figure 1). Brain homogenate from an infected RAG/STAT-/- mouse was passed into 129 wild-type or IFNαβγR-/- mice before and after filtration. A full work-up was performed on mice from passages 1 and 2, including histopathology, electron microscopy, standard clinical virology and microbiology work-ups, as well as special stains of histology sections (GMS, AFB [acid-fast bacilli], Gram stain). All of these failed to reveal the nature of the pathogen.

The pathogen is more virulent in mice lacking both the interferon αβ (IFNαβ) and the interferon γ (IFNγ) receptors (IFNαβγR-/-, [2]) than in wild-type mice (see below) and it passes through a 0.2μm filter (see above and Fig. 1A). The pathogen does not appear to cause cytopathic effect on HeLa cells, Vero cells or murine embryonic fibroblasts (including those lacking IFN receptors or STAT1). These data suggest that a previously unknown IFN- sensitive but non-cultivatable pathogen that was < 0.2μm in size was present in diseased mice.

IDENTIFICATION AND SEQUENCING

To identify the new pathogen a previously published representational difference analysis protocol (RDA) was used (See Pastorian et al., *Anal. Bicochem.* 283:89-98 (2000), which is hereby incorporated in its entirety). Double-stranded cDNA (dsDNA) from the brain of an infected IFNαβγR-/- mouse at passage 2 (Fig. 1) was prepared, digested with restriction enzymes, and ligated to adaptors containing PCR primer sequences (tester) (see Pastorian protocol for sequences of RDA primers). Control dsDNA lacking linkers was prepared concurrently from a control brain (driver). Serial rounds of subtractive hybridization of tester in the presence of excess driver followed by PCR amplification of tester-specific sequences were performed to generate difference products (DP) one through four (DP1-DP4). DP3 and DP4 were cloned and sequenced. Three of 24 clones from DP3 and ten of 48 clones derived from DP4 had significant homology to multiple caliciviruses (data not shown). These RDA clones spanned the Norwalk virus genome (Fig. 2A), but were not identical to any known full or partial calicivirus sequence, demonstrating that we had identified a novel calicivirus. This new virus is referred to herein as murine Norovirus-1 (MNV-1).

To determine the relationship of MNV-1 to other caliciviruses, the MNV-1 genome was cloned and sequenced from cDNA of an infected mouse brain using a combination of 5' and 3' RACE and PCR (Fig. 2A). Sequencing was performed in both directions with 10-fold redundancy to obtain a consensus sequence with the exception that the 5' 15 nucleotides were obtained from a single clone. The assembled genome included 7726bp of unique sequence plus a 3' polyA tail, and contained the expected three ORFs conserved across the *Caliciviridae* (Fig. 2B). Phylogenetic analysis using the CLUSTAL W algorithm, and other algorithms including PAUP, aligning either the complete genome sequence (Fig. 2C) or the capsid protein sequence (Fig. 2D) of MNV-1 with corresponding sequences from members of the four calicivirus genera and several members of the *Picornaviridae* family revealed that MNV-1 is a *Norovirus* that does not cluster

within previously identified genogroups (Fig. 2C, D)(Green KY JID 181 S322-330). Therefore, it is proposed that MNV-1 is exemplary of a new *Norovirus* genogroup.

Thus, disclosed herein is a pathogen that infects mice, referred to herein as Murine Norovirus-1 (MNV-1). MNV-1 is both a unique norovirus, and is the first member of a new genogroup of Noroviruses. An exemplary sequence for the MNV-1 virus and genogroup is provided as SEQ ID NO:1, which is a consensus sequence representative of the full length MNV-1 genome as determined from a series of clones derived by PCR or RACE analysis from RNA derived from the brain of an infected mouse. Thus, one embodiment comprises an isolated RNA sequence as shown in SEQ ID NO:1. An additional embodiment comprises sequences of MNV-1 isolates that vary from the sequence in SEQ ID NO:1 by an amount determined by both sequence analysis and current understanding of the relatedness of different caliciviruses (see below). One embodiment comprises the viruses related directly to MNV-1 as viral quasispecies. Another embodiment comprises other members of the MNV-1 genogroup of which MNV-1 is the defining member. The criteria for viral quasispecies and viral genogroup are defined below, and serve to specifically set criteria for the MNV-1 embodiments described herein.

RNA viruses vary during infection due to errors made by the viral RNA-dependent RNA polymerase. Thus, MNV-1 (a positive-strand RNA virus) may be expected to vary during replication into a quasispecies comprising multiple viruses with sequences closely related to, but not identical to, the sequence of the original infecting virus. Thus, some embodiments of MNV-1 include viruses with sequences that vary from the sequence provided in SEQ ID NO:1 by an amount consistent with variation within a calicivirus quasispecies. The level of variation from the MNV-1 consensus SEQ ID NO:1 that still is considered by those skilled in the art to be the same virus (since these viruses always exist as quasispecies) is 5-7% (Radford et. Al. Veterinary rewcord Jan 29, 2000 pp117 on, Radford et al Vet Record October 20 2001 pp 477 on). Thus, an embodiment comprises the MNV-1 viral quasispecies of sequences that vary from our initial consensus sequence (SEQ ID NO:1) by no more than 5%. It has been confirmed that there is significant variance in MNV-1 nucleotide sequence even within a single infected animal (Fig. 3). To show this, a portion of the primary data from which the 10-fold redundant consensus sequence SEQ ID NO:1was derived is presented. It was found that over the highly conserved ORF2 region, there are multiple sites at which there is sequence variation (Fig. 3A). A portion of the sequence data is presented in Fig 3B for a region within which sequence variation was found. The frequency of variation at the sites shown in boxes is greater than that observed at multiple

other sites (e.g. the remainder of the sequence in Fig. 3B), showing that these variations represent true biological variation rather than PCR artifacts or sequencing errors. Thus, MNV-1 does exist as a quasispecies.

Further embodiments comprise viruses with an amount of variance from SEQ ID NO:1 that is consistent with variation within a genogroup, and less than the variation observed between genogroups. For caliciviruses, genogroup and genus definition has been developed and officially set by the International Committee on the Taxonomy of viruses (ICTV) and research in the field has led to definitions of the amount of variation in sequence that is expected within a single genogroup as opposed to between viruses of different genogroups (K. Y. Green et al JID 2000 S322-330). Because nucleotide sequences can vary without causing variation in amino acid sequence, relatedness at the nucleotide level is a preferred method for distinguishing between genogroups or within a quasispecies (see above). Nucleotide identity within a genogroup of Noroviruses has been established as greater than 80% within the highly conserved capsid protein (ORF 2) gene (J. Vinje et al Arch Virol (2000) 145:223—241). Thus, viruses that differ by more than 20% at the nucleotide level from a member of a genogroup (in this case from the MNV-1 sequence in SEQ ID NO:1) are not members of the genogroup. Nucleotide identity between genogroups is 64%-78% or less. Therefore, the genogroup to which MNV-1belongs comprises viruses that vary by no more than 20% from the MNV-1 sequence within the capsid region. Similar reasoning applies to other conserved regions of the genome including the RNA dependent RNA polymerase gene. Therefore, our use of the capsid sequence for the definition of genogroup is standard.

Further embodiments include RNA sequences that are at least about 80% identical to SEQ ID NO:1, where the % identity is determined using Vector NTI AlignX program. Other embodiments include an isolated DNA sequence, or fragments thereof, identical to or complementary to SEQ ID NO:1, and isolated DNA sequences at least about 80% identical to or complementary to SEQ ID NO:1. Further embodiments comprise sequences between 80% and 100% identical to SEQ ID NO:1, and sequences complementary thereto.

Additional embodiments comprise fragments of any of the above mentioned sequences, such as may be used, for example, as primers or probes. Examples of such sequences include the primers listed in legends to Fig. 8 and 10 that were used to detect virus infection in animals by nested PCR (Fig. 10) or to determine the amount of MNV-1 genome in a tissue by the use of real time PCR (Fig. 8). These primers will be useful for detection of MNV-1 infection in

commercially bred mice and for quantitation of MNV-1 in tissues after trials of antiviral agents or vaccines. Such primers and probes are selected such that they are substantially complementary to a target sequence, wherein the target sequence consists of coding sequence of MNV-1. Here, substantially complementary means that the primer or probe is sufficiently complementary to the target sequence that it will hybridize to the target sequence under highly stringent conditions. As used herein, highly stringent conditions are as defined in the nested and real time PCR protocols exemplified in figures 8 and 10. For hybridization in blots as opposed to PCR reactions, stringent refers to: hybridization at 68 degrees C in 5x SSC/5x Denhardt's solution/1.0% SDS, and washing in 0.2x SSC/1.0% SDS at room temperature. Such probes and primers are useful, for example in various assays for detecting the presence of MNV-1 (Fig. 10) and determining how much MNV-1 is in a particular sample (Fig. 8). Other assays for which such primers or portions of MNV-1 sequence would be useful include Northern and Southern hybridization blot assays, additional PCR assays (e.g. degenerate PCR using primers with degenerate nucleotides at specific sites within the PCR primer to detect viruses within the MNV-1 genogroup but not identical to the MNV-1 sequence in SEQ ID NO:1), transcription-mediated amplification assays and the like, and as positive controls and internal standards for commercial assays to detect the presence of MNV-1 in mice or after treatment with anti-viral agents or vaccines.

A feature that distinguishes the human *Noroviruses* from the *Sapoviruses* are the cup-shaped depressions on the virion surface that gave the calicivirus family its name (*calyx* = cup in Latin). *Sapovirus* capsids show these characteristic cup-shaped depressions by electron microscopy (EM), while *Norovirus* capsids have a feathery appearance. To visualize MNV-1 virions, MNV-1 was purified from the brain of an infected IFNαβγR-/- mouse on CsCl gradients (Fig. 4). Gradient fractions containing MNV-1 genome were identified by RT-PCR (Fig. 4A), revealing a buoyant density of MNV-1 of 1.36g/cm^3 +/- 0.04 g/cm^3 (n=3 experiments), in close agreement with the published buoyant densities of *Noroviruses* (1.33-1.41g/cm^3). Analysis of these gradient fractions by EM revealed particles with a diameter of 28-35 nm (Fig. 4B), similar to the known size (26-32 nm) of *Norovirus* particles in negatively stained preparations. The particles were icosahedral and had the same feathery surface morphology as *Noroviruses* but lacked the cup-like depressions characteristic of *Sapoviruses*. Gradient fractions prepared from mock-infected brain did not contain these particles (data not shown).

To test the pathogenicity of MNV-1, mice were infected i.c. with CsCl gradient purified MNV-1. These virions were infectious since 18/18 RAG/STAT mice inoculated with them died,

while 18 of 18 mice inoculated with gradient fractions prepared from a mock-infected brain survived (Fig. 4C). Mice inoculated with gradient-purified virions showed encephalitis, meningitis, cerebral vasculitis, pneumonia, and hepatitis (data not shown). This mortality rate and pathology was similar to that observed previously in mice inoculated with unpurified brain homogenate (Fig. 4C and data not shown). The presence of disease in mice inoculated with CsCl-purified MNV-1 demonstrates that MNV-1 is the causative agent of the disease initially detected and passed (Fig.1).

The MNV-1 genome comprises three open reading frames (ORFs). Analysis of the predicted coding sequence of ORF1 indicated a polyprotein with a molecular weight (MW) of 180.7kDa and revealed the presence of multiple conserved motifs shared by caliciviruses and picornaviruses (Fig. 2B). ORF2 is separated from ORF1 by 32nt and starts in the –1 frame relative to ORF1. It encodes a 542 aa protein with a calculated MW of 58.9kDa and an isoelectric point of 5.19. When this gene was expressed in a recombinant baculovirus, virus-like particles (VLPs) were found in the supernatant of infected cells, demonstrating ORF2 encodes the capsid protein (Fig. 6). These VLPs provide a reagent for analysis of MNV-1 infection (see below). The predicted ORF3 encodes a basic protein (pI of 10.22) with a calculated MW of 22.1kDa that overlaps by 2nt with ORF2 and is expressed in the +1 frame relative to ORF1 but the –2 frame relative to ORF2.

Thus, further embodiments comprise the amino acid sequences encoded by ORF1, ORF2 and ORF3. These amino acid sequences are shown in SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4, respectfully. Additional embodiments comprise amino acid sequences that are encoded by viruses that vary from SEQ ID NO:1 by no more than 20% at the nucleotide level as defined above. The protein translation of such sequences will vary on a percentage basis depending on the placement of nucleotides within codons and the frequency of amino acids coded for by single versus multiple three base pair codons used by the translational machinery. Therefore the extent of variation of these embodiments is properly determined by defining the extent of total nucleotide variation accepted as defining the MNV-1 genogroup. Some embodiments comprise the nucleotide sequences that encode each of the amino acid sequences of SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4, including degenerate variants that encode those amino acid sequences. Additional embodiments comprise the nucleotide sequences of ORF1, ORF2 and ORF3 of MNV-1.

Additional embodiments include vectors capable of expression of any of the proteins encoded by MNV-1 or their variants as defined above. Examples of suitable vectors include baculovirus vectors, alphavirus vectors (e.g. Sindbis virus vectors, VEEV replicons), retroviral vectors, plasmids within which expression is driven from eukaryotic promoters, plasmids that generate short RNA sequences suitable for gene inactivation by RNAi technology, plasmids in which the presence of an RNA polymerase transcribes MNV-1 sequences (including the entire sequence) in order to provide RNA (including up to full length infectious RNA) for analysis or transfection into cells. Infectious RNA is defined as RNA, which, on transfection into eukaryotic cells, gives rise to intact infectious virus. Portions of the genome may also be expressed in this fashion for the generation of viral proteins or for analysis of the processing of MNV-1 viral proteins for the purpose of developing assays for identification of steps in viral replication that may serve as drug targets. Additional uses of expression vectors include generation of cells expressing viral proteins in a stable fashion for the purpose of screening anti-viral antibodies or for providing positive controls for assay for detection of anti-viral antibody in the serum.

As discussed above, expression of the capsid protein, i.e., the protein encoded by the sequence of ORF2, results in the formation of virus-like particles (VLPs). Thus, some embodiments comprise methods of producing VLPs. Such methods comprise transfecting a cell or animal with a vector that encodes the MNV-1 capsid protein, and recovering VLPs, or expression of the capsid protein from within the baculovirus genome such that the capsid protein is produced in insect cells infected with the baculovirus expressing the capsid protein. Further embodiments comprise MNV-1 VLPs. VLPs are useful, for example, for isolation of antibodies, analysis of the epitopes that antibodies recognize, and for cryo-EM and X-ray crystallography and other methods for determining the three dimensional structure of the MNV-1 capsid. VLPs may also be studied for potential use as a vaccine. In this setting they may be useful for mapping the specific conformational epitopes recognized by anti-viral antibodies and the specific peptides recognized by antiviral CD4 and CD8 T cells.

ANTIBODIES

Some embodiments comprise antibodies that bind specifically to any of the various proteins encoded by the MNV-1 genome. Methods for producing antibodies are known in the art. Such antibodies may be either monoclonal or polyclonal. Antibodies can be used in various assays, such as for example ELISA assays, to detect the presence of MNV-1in a sample. Samples

include for example serum, saliva, feces, and tissues. In addition, antibodies may be utilized in methods for preventing lethal MNV-1 infection and studied for potential use as vaccines or anti-viral therapeutics.

An example of the use of antibodies and antibody detection assays is the demonstration that seroconversion can be detected by ELISA of serum using MNV-1 VLPs as the target antigen bound to the ELISA plate (Fig. 7). A further example is the demonstration that MNV-1 infection can be detected in specific cells by using immunohistochemistry to detect the binding of MNV-1 specific antibodies to infected cells (Fig. 9). This type of use may also be employed for detecting binding of virus to cells by FACS analysis. This in turn will provide an opportunity to identify the receptor for MNV-1. Identification of the MNV-1 receptor on the cell surface may provide important targets for anti-viral drug development. In addition, antibodies will be used for immunofluorescence and in-situ detection of virus infected cells.

SMALL ANIMAL MODEL

The discovery of the first murine *Norovirus* provides the first small animal model for development and testing of pharmaceuticals and vaccines for treatment and prevention of a major human disease. This presents an opportunity to answer important questions regarding the pathogenesis of *Norovirus* infections, to determine the role and mechanisms of immunity in either protection against infection or immunopathology, to identify novel therapeutic targets for treatment of human calicivirus disease, and to better understand how innate immunity can control enteric virus infection. The mouse model can also be used in methods to identify agents that alter calicivirus infection and disease.

The course of human *Norovirus* infection strongly suggests that symptoms are caused by acute infection. Prominent amongst the clinical manifestations are vomiting and diarrhea with a mean incubation period of 24 hours and duration of 24-48 hours. Understanding of the viral and host mechanisms involved in the induction and clearance of human *Norovirus* disease is rudimentary. Acquired immunity can play a role in *Norovirus* resistance, but may not explain why certain individuals get severe disease while others do not. It may be that long-term immunity can be achieved, and the use of the MNV-1 virus in a small animal model provides the first opportunity to define such possible mechanisms. Infected individuals can develop short-term immunity to homologous virus, but the development of long-term immunity is questionable. An unexpected inverse relationship between pre-challenge antibody levels and susceptibility to

infection has been reported in some studies (Parrino, T.S., et al., N. Engl. J. Med. 297:86-89, 1977; Johnson, P.C. et al., J. Infect. Dis. 161:18-21, 1990; Okhuysen, P.C. et al., J. Infect. Dis. 171:566-569, 1995), while others have reported that circulating antibody does correlate with resistance to calicivirus infection (Lew, J.F. et al., J. Infect. Dis. 169:1364-1367, 1994; Ryder, R.W. et al., J. Infect. Dis. 151:99-105, 1985; Nakata, S. et al., J. Infect. Dis. 152:274-279, 1985). This controversy has led to studies showing that non-immune host factors, such as blood groups, influence susceptibility to infection(Hutson, A.M. et al., J Infect. Dis. 185:1335-1337, 2002). The discovery of MNV-1 provides a small animal model for the study of *Noroviruses*.

One embodiment is therefore the use of mice infected with MNV-1 as an approach to identifying the efficacy of vaccines or therapeutic agents. Mice would be infected with the newly discovered virus, and then treated with candidate agents and the outcome of the infection monitored using ELISA (Fig. 7), quantitative real time PCR for the viral RNA (Fig. 8), immunohistochemistry (Fig. 9), lethality (Fig. 5), or in situ hybridization to monitor the course of infection. Similarly, another embodiment is the use of mice infected with MNV-1 to test the efficacy of vaccination protocols against the virus. In this case, different vaccine preparations (including vectors expressing portions of the new virus genome or proteins from the virus or from human noroviruses that cross react with proteins from the mouse virus) would be administered to infected mice and the effect of vaccination on the course of the infection monitored using ELISA (Fig. 7), quantitative real time PCR for the viral RNA (Fig. 8), immunohistochemistry (Fig. 9), lethality (Fig. 5), or in situ hybridization. As it is not practical to perform such experiments in humans, the capacity to perform these types of screens for in vivo efficacy of therapeutics or vaccines is not possible without the use of this newly described virus.

In addition, the discovery of MNV-1 and the generation of a consensus sequence will enable construction of an infectious clone for MNV-1. One embodiment is therefore generation of such an infectious clone from the current cloned and sequenced genome or from sequences that vary within the limits described above for the MNV-1 quasispecies or MNV-1 genogroup. Such a clone can be used to develop various screening assays for MNV-1 antiviral agents and targets for antiviral drug development and vaccines for prevention of infection or antibodies for therapy of disease,and also may be used to study certain aspects of the viruses infection cycle including binding, entry, uncoating, negative strand RNA synthesis, positive strand RNA synthesis, subgenomic RNA synthesis, synthesis of structural and non-structural proteins, viral assembly and viral egress to be studied and used to develop screens for antiviral drugs that might have uses

in preventing or treating Norovirus induced disease. In addition, placement of portions of human Noroviruses into an infectious clone for MCV-1 (e.g. substituting proteins such as the capsid of RNA polymerase of the human virus into the mouse virus infectious clone) will allow the murine virus to be humanized and potentially still used in mice. This will allow screening of therapeutic agents that target the functions of human norovirus proteins in an animal model. This is possible only through the combined use of an infectious MNV-1 clone as a vector for expressing functional proteins and a small animal model which allows assessment of the effects of therapeutic agents or vaccines on the course of infection with such "humanized" forms of the mouse calicivirus MNV-1.

In addition, the use of the newly discovered MCV-1 virus in mice with different immune deficiencies will allow identification of host proteins that play a role in control of Norovirus infection. An example of this is the detection of the critical role of STAT-1 in resistance to MNV-1 infection (Working Example 14, Fig. 5). Identification of such host proteins could allow development of targeted therapeutic agents that enhance specific parts of the host immune response as a way to treat or prevent Norovirus disease. Such embodiments include, for example, use of the virus in mice with deficiencies in specific parts of the immune system in order to identify mice that have increased susceptibility or increased resistance to infection by MCV-1. Such embodiments would be useful for identifying immune protective or immunopathologic aspects of the host response and thereby inform searches for vaccines or therapeutic agents that could prevent or treat Norovirus infection. An example would be targeting enhanced STAT-1 function, based on the experiments in Figure 5, for prevention of Norovirus disease in humans.

WORKING EXAMPLES

Example 1: Generation of MNV-1 stock

After identification of MNV-1 in RAG/STAT and IFNαβγR-deficient mice, a brain homogenate from an IFNαβγR-deficient mouse at passage 3 was used for i.c. inoculations of 17 additional IFNαβγR-deficient mice. Brains of infected mice were harvested 3 days post-infection and homogenized in PBS. Homogenates were centrifuged at low speed and filtered through a 0.2μm filter and the resulting supernatant was used in subsequent infections. For control experiments, brain homogenates of mock-infected mice were prepared similarly after injection of mice with uninfected mouse brain homogenate. (See Fig. 5).

Example 2: Purification of MNV-1 virions

Homogenate from one MNV-1 infected brain was used for purification of MNV-1 virions while a mock-infected mouse brain was used as a control (Fig. 4). Homogenized brain was subjected to a cycle of freeze/thaw and two low speed centrifugations before filtration through 0.22μm filter. Supernatants were centrifuged at 90,000 x g for 2hrs and the resulting pellets were incubated for 30min at 37C in 1ml 1% Na-deoxycholate. The resulting material was mixed with CsCl to a final density of 1.36g/cm^3 and centrifuged for 40hrs at 150,000 x g. Gradients were fractionated, their density determined with a refractometer, and dialyzed against a buffer containing 0.01M Tris-HCl, 0.15M NaCl, 1mM $CaCl_2$, and 0.05M $MgCl_2$. (See Fig. 4).

Example 3: RNA isolation, cDNA synthesis, and RDA

Total RNA was isolated from a MNV-1 infected mouse brain using Trizol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Double-stranded cDNA for use in RDA was synthesized from total RNA using the Superscript Choice System for cDNA synthesis (Invitrogen, Carlsbad, CA) and a combination of random hexamers and oligo dT primers. Single-stranded cDNA for quantitative PCR was generated using Supercript II (Invitrogen, Carlsbad, CA) following the manufacturer's recommendations. RDA was performed as described by Pastorian *et al.* (Anal. Biochem. 283:89-98, 2000) with the following modification. The QIAquick PCR purification kit (Quiagen, Valencia, CA) was used to remove unincorporated nucleotides and small cDNA species. Difference products from rounds 3 and 4 were cloned into the pGEM-T vector system (Promega, Madison, WI) following the manufacturer's instructions. Bacterial colonies were grown up and inserts were PCR amplified for sequencing. (See Fig. 2, 3).

Example 4: RT-PCR and quantitative PCR.

RT-PCR was performed with primers 445/1/AS6 (TCCAGGATGACATAGTCCAGGGGCG)(SEQ ID NO:5) and 445/1/S6 (TGGGATGATTTCGGCATGGACAACG) (SEQ ID NO:6) using the Titanium one-step RT-PCR kit (Clontech, Palo Alto, CA) following manufacturer's recommendations. Quantitative PCR (Fig. 8) was performed with primers ORF1/RT1/S (cagtgccagccctcttat) and ORF1/RT1/AS2 (tcctcctcatcaagggac) that amplify a 132 bp segment of ORF1 outside of the predicted subgenomic RNA. This assay has a sensitivity of 100 viral genomes/μg cellular RNA or about 1 MNLV-1

genome per 1720 cell equivalents of RNA (estimating 1μg cellular RNA/172,000 cells). The assay linearly quantitates genome over at least a 6-log range. (See Fig. 8).

Example 5: Cloning of the MNV-1 genome.

A combination of PCR and RACE was used to clone the MNV-1 genome (Fig. 2A). For internal sequence information, primers were constructed based on sequence information obtained through RDA and used to amplify and clone larger pieces of MNV-1 from 1st strand cDNA from a RAG/STAT mouse brain (passage 3). These PCR products were cloned into the pGEMT vector (Promega, Madison, WI) and universal M13 forward and reverse primers used for sequencing. Primer walking was applied when necessary. For the 5' and 3' ends of MNV-1, RACE was performed with the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA) using total RNA from the same RAG/STAT mouse brain (passage 3) as starting template. These products were cloned and sequenced as outlined above. A consensus sequence with at least 10-fold redundancy (except for the 5'end, see below) was constructed using the VectorNTI contig program. The 5'end of the genome was difficult to clone and consequently the first 15 nucleotides are based on a single sequence, possibly explaining why there is no start codon close to the 5'end as is expected based on comparison with other *Noroviruses*. (See Fig. 2).

Example 6: Cloning and expression of the MNV-1 capsid protein in bacteria.

The MNV-1 capsid protein was PCR amplified from first strand cDNA from a RAG/STAT mouse brain (passage 3). The following primers C-pET1 (GTGGTGCTCGAGTGCGGCCGCAAGCTTTATTATTGTTTGAGCATTCGGCCTG) (SEQ ID NO:7) and N-pET1 (ATCCGAATTCTAGATG**CACCACCACCACCACCAC**ATGAGGATGAGTGATGGCGCAG) (SEQ ID NO:8) containing HindIII and EcoRI restriction sites (underlined), respectively, and a 6 Histidine N-terminal tag (bold) were used in a 2 step PCR reaction (5 cycles 50C, 30 cycles 60C) in the presence of 5% DMSO. The resulting PCR product was ligated into a PCR blunt cloning vector (Zero Blunt PCR Cloning kit, Invitrogen, Carlsbad, CA) and transformed into DH5α $CaCl_2$ competent cells (Invitrogen, Carlsbad, CA). DNA was isolated from the resulting clones and diagnostic restriction digests followed by DNA sequencing confirmed the presence and sequence of the insert. The insert was cloned into the bacterial expression vector pET-30a (+) (Novagen, Madison, WI) using the EcoRI and HindIII restriction sites. Next, BL21 (DE3)

competent cells were transformed and protein was expressed following the manufacturer's protocol (Novagen, Madison, WI).

Example 7: Purification of bacterially expressed MNV-1 capsid protein.

Following a 2 hour expression, protein was purified from inclusion bodies of bacterial cells using the BugBuster protein extraction reagent (Novagen, Madison, WI). His-tagged capsid protein was isolated from remaining protein by nickel column chromatography (Ni-NTA His Bind Resin, Novagen, Madison, WI) in the presence of 8M urea and protease inhibitors (protease inhibitor cocktail set III, Novagen, Madison, WI). Samples were dialyzed against 25mM phosphate buffer (pH 6.0) and the purity of each preparation was assessed by SDS-PAGE and silver staining (Silver stain Plus kit, Biorad, Hercules, CA).

Example 8: Generation of antisera in rabbits.

Rabbit sera was produced through Cocalico Biologicals, Inc. (Reamstown, PA). Basically, rabbits were injected with 100μg bacterially expressed capsid protein in CFA (complete Freund's adjuvant) and boosted after a month once every month with 50μg protein in IFA (incomplete Freund's adjuvant). Production bleeds were collected a week after each boost and before the start of injections. The same procedure is being used for generation of antibodies directed against virus-like MNV-1 particles.

Example 9: Cloning and expression of the MNV-1 capsid protein in baculovirus.

The MNV-1 capsid protein was cloned into the baculovirus expression vector in an analogue way to the cloning into the bacterial expression vector. The following primers were used for initial 2 step PCR amplification (4 cycles at 50C, 30 cycles at 64C) of the MNV-1 capsid protein:

N-Bac1 (CGGAATTCGGATGAGGATGAGTGATGGCGCA)(SEQ ID NO:9), C-Bac1 (TCTCGACAAGCTTTTATTGTTTGAGCATTCGGCCT)(SEQ ID NO:10). The same restriction sites, EcoRI and HindIII (underlined) were used for cloning into the pFastBac1 vector (Invitrogen, Carlsbad, CA). Recombinant baculoviruses were made using the Bac-to-Bac Expression system (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Briefly, the recombinant vector plasmid containing the MNV-1 capsid protein was transformed into DH10Bac *E.coli* cells allowing for transposition of the gene of interest into the bacmid genome.

Clones containing recombinant bacmids were identified by antibiotic selection and disruption of the *lacZ* gene. Recombinant bacmid DNA was then used for transfection of Sf9 insect cells. Recombinant baculoviruses were amplified for several rounds on Sf9 or Sf21 cells (Invitrogen, Carlsbad, CA) before infection of High-Five cells (Invitrogen, Carlsbad, CA) for protein expression. High-Five cells were infected for 5-7 days and supernatant were collected for purification of MNV-1 VLPs. Initial preparations were screened for the presence of VLPs by negative staining electron microscopy. VLPs were identified in the supernatants of several isolates (Fig. 6C). Two isolates with the highest amount of protein expression were chosen for further experiments. The amount of protein expression in each preparation was analyzed by SDS-PAGE and immunoblotting (Fig. 6A).

Example 10: Purification of MNV-1 VLPs.

MNV-1 VLPs are purified from the supernatant of infected High-Five cells 7 days post-infection (Fig. 6D). The purification protocol is based on Leite *et al.* (Arch Virol, 1996, 141:865-875), which is hereby incorporated by reference. Briefly, protein in the cell supernatant is being precipitated using PEG 8000, and particles are purified using CsCl gradients. VLPs are dialyzed against 25mM phosphate buffer, pH 6.0. Details of the protocol are being optimized at this point.

Example 11: Use of VLPs, potential and actual targets of VLPs.

VLP-containing insect cell supernatants are being used for ELISA to screen mouse sera (see ELISA below). VLPs will be used to generate rabbit antisera. Their role as potential vaccine will be investigated. They will also be used for three-dimensional structure determination of the MNV-1 capsid.

Example 12: ELISA Assay

This assay can be used to screen mice capable of eliciting an antibody response (Fig. 7). The assay was optimized for a maximal signal-to-background ratio. VLP-containing insect cell supernatants are used as antigens for coating ELISA plates over night at 4C. Plates are blocked for two hours at 37C with 3% BSA and washed with 0.15M NaCl + 0.05% Tween 20. Sera from mice are diluted 1:100 and incubated for 1 hour at 37C. After washing, wells are incubated for two hours at 37C with a 1:1000 dilution of peroxidase-conjugated AffiniPure goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Plates are developed

after another round of washing by addition of the substrate 2,2'-Azinobis 3-ethylbenzthiazoline sulfonic acid (ABTS, Sigma-Aldrich Corp., St. Louis, MO) for 10min, the reaction is stopped using 0.2N phosphoric acid, and quantified by reading the absorbance at 415nm.

Example 13: Nested PCR Assay

This assay can be used to screen tissues of immunocompromised mice with no antibody response (Fig.10). RNA is isolated from the tissue(s) of interest and 1st strand cDNA is being made (see above). To sets of primers were designed with the following sequences: outer sense primer CCAAAAGCCAATGGCTCTGA (SEQ ID NO:11), outer antisense primer AGTTGAATGGGCTCCAGGGT (SEQ ID NO:12), inner sense primer CCGCCGGGCAAATTAACCAA (SEQ ID NO:13), inner antisense primer AGGTGGGCAAGGTAGGGGTTA (SEQ ID NO:14). Each reaction contained 2µl of first strand cDNA and a final concentration of 1µM sense and antisense primer, 2.5mM $MgCl_2$, 0.2 mM dNTPs, and 1.25 unit *Taq* DNA Polymerase (Promega, Madison, WI) in 1x buffer (*Taq* DNA Polymerase 10X Reaction Buffer without $MgCl_2$, Promega, Madison, WI). PCR was performed for 45 cycles for the 1st round, and 30 cycles for the 2nd round with the following setting: heating 2min 94C, cycle for 30sec 94C, 30sec 60C (annealing), and 30sec 72C (extension), with a final extension step of 10min 72C. Two µl product from the 1st round are used in the 2nd round using the same overall set-up. Products are analyzed by agarose gel electrophoresis.

Example 14: Use of MNV-1 infected mice as small animal model of Norovirus infection

To determine whether T and B cell mediated immunity is required for resistance to MNV-1, wild-type and RAG1-/- mice were infected by the i.c. route and followed for 90 days (data not shown). Surprisingly, MNV-1 infection does not kill RAG1-/- mice (n=20) after direct i.c. inoculation even though these mice are typically highly susceptible to infection with a range of different viruses. The finding that RAG-/- mice are resistant to lethal disease argues that typical adaptive responses are not required for protection from lethal disease. This finding may explain in part contradictory conclusions as to the importance of antibody in resistance to *Norovirus* disease. While B and T cell responses are not required for resistance to lethal infection, it may be that pre-existing immunity influences the pathogenicity of MNV-1.

Alternatively, the presence of immune cells may contribute to disease induced by MNV-1 as is seen for lymphocytic choriomeningitis virus.

Together with a course of clinical illness too brief to allow typical adaptive responses, these studies in RAG-/- mice beg the question of whether innate rather than acquired immunity is critical for resistance to calicivirus infection. We therefore inoculated a variety of mouse strains lacking components of the innate immune system with MNV-1. The peroral (p.o.) and intranasal (i.n.) routes were tested in addition to the i.c. route since the physiologic routes of infection for human caliciviruses are oral and respiratory. Mice lacking the IFNαβ receptor or the IFNγ receptor were no more susceptible to lethal infection than wild-type controls (Fig. 5A). In contrast, mice lacking both IFNαβ and IFNγ receptors were more susceptible to lethal infection than congenic controls after either i.c. or i.n. inoculation with MNV-1 (Fig. 5B). These data demonstrate that the IFN receptors can compensate for each other in resistance to MNV-1 infection such that only deficiency in both receptors leads to lethality. Mice deficient in inducible nitric oxide synthetase (iNOS) or in the RNA activated protein kinase PKR, two IFN regulated proteins with antiviral properties, were also resistant to lethal MNV-1 infection after i.c. or p.o. inoculation (Fig. 5A).

Since deficiency in both IFN receptors is required to predispose to lethal MNV-1 infection, we reasoned that a component of the innate immune system that can be activated by either the IFNαβ or the IFNγ receptor was critical for MNV-1 survival. We therefore tested the hypothesis that the latent cytoplasmic transcription factor STAT1, which is shared by both the IFNαβ and IFNγ signaling pathways, was critical for resistance to calicivirus infection. STAT1 deficiency resulted in lethal MNV-1 infection in mice with T and B cells (STAT1-/-, Fig. 5C), mice lacking T and B cells (RAG/STAT, Fig. 5D), and mice lacking PKR (PKR-/-STAT-/-, Fig. 5E) by all routes analyzed. Thus STAT1 is the first host component identified as essential for resistance to lethal *Norovirus* infection, and is required for survival even when T and B cells are present.

Having identified STAT1 as essential for calicivirus resistance, we then investigated the relationship between the interferon receptors and STAT1. No statistically significant differences were found in the survival of IFNαβγR -/- and STAT1-/- mice after either i.c. or i.n. inoculations. However after p.o. inoculation, deficiency of STAT1, but not deficiency in both IFNαβ and IFNγ receptors, led to lethal infection (see Fig. 5B and C). This might suggest that STAT1 has IFN receptor-independent effects that are critical for *Norovirus* resistance. Supporting this are

findings that the biological effects of STAT1 do not completely overlap with those of the IFN receptors during viral infection since there are both STAT1-independent antiviral effects of the IFN receptors, and IFN receptor-independent effects of STAT1.